REMARKS

Status of the Application

This application is being filed as a RCE. Prior to the present amendment, claims 23 – 31 and 33 - 46 were pending. With entry of the instant amendment, claims 23 – 27, 29 - 31, 33 - 40 and 42 – 51 are pending. Claims 23, 27, 29, 30, 38, 39, 40, 42 and 43 have been amended, claims 28 and 41 are canceled, and claims 47 - 51 are new. New matter has not been introduced by the amendment. For the Examiner's convenience a clean set of claims has been attached as Appendix II.

Status of the Claims

Claims 23, 27, 38, 39, 40, 42 and 43 are independent claims. In the Office Action dated August 13, 2002, paper number 32, the Examiner finally rejected claims 23 – 31, 33 – 38, 41, 42 and 44 - 46 and objected to claims 39, 40 and 43. It was indicated in the Office Action that claims 39, 40 and 43 would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims. Claim 39 depended from claim 38, claim 40 depended from claim 23, and claim 43 depended from claim 42. These claims are directed to a mutant host cell having a specific growth rate on glucose as a sole carbon source of about $0.8h^{-1}$. Each claim has been rewritten in independent form.

Amended claim 23 recites that the Pts-/Glu+ phenotype is due to the deletion or inactivation of all or substantially all of a gene selected from the group of *ptsI*, *ptsH* and *crr*. Essentially the limitation of now canceled claim 41 has been incorporated into claim 23. Claim 27 has been amended to delineate the steps of the method more clearly. Step (a) recites obtaining a host cell mutant having certain characteristics and step (b) recites culturing the host cell mutant. The claim as amended also includes the recitation of now canceled claim 28 (the Pts- phenotype is due to the deletion or inactivation of all or substantially all of a gene selected from the group of *ptsI*, *ptsH* and *crr*). Amended claim 30, which depends from claim 27, has been modified to provide proper antecedent basis. The phrase "selected host cell" as been replaced with the phrase "host cell mutant". Amended claim 38 recites a method for obtaining a Pts- /Glucose^{+}, galactose permease requiring-mutant cell, which includes culturing the mutant host cell under continuous culture conditions and then selecting mutant host cells which grow on glucose at a specific growth rate of at least $0.4h^{-1}$. The disclosure includes numerous references to growing the Pts- cells in a chemostat, and reference is made to page 10 wherein

it is stated, "The use of a chemostat as described herein allowed the isolation of a collection of spontaneous mutants that can grow on glucose with different growth rates.". Additionally, original claim 19 was directed to selecting cells in a continuous culture. It is generally known by one of ordinary skill in the art that a chemostat is a method used for continuous culture. Claim 42 has been amended to more clearly delineate the steps of the method. Previous step b) directed to "optionally recovering said compound" has been omitted from the amended claim and presented as a dependent claim and reference is made to new claim 50.

New claims 47 and 48 correspond to pending claims 25 and 24, respectively. However, the new claims depend from allowable claim 40. New claim 49 depends from claim 38 and further defines the specific growth rate of the mutant cell as being at least 50% of the host cell of step a). Support is found at page 14 of the disclosure. New claim 51 depends from claim 43 and is directed to the further step of recovering the desired compound.

Rejections under 35 U.S.C §103(a)

The Examiner has rejected claims 23, 27, 38, 41, 45, and 46 as being unpatentable over Saier et al. (Saier) and as being unpatentable over Ingrahm et al. (Ingrahm). Claim 23 is directed to the mutant cells per se. Claims 27, 38 and 42 are independent method claims and each claim recites a specific growth rate of at least 0.4/hr.

While Applicants disclose Pts- mutants have a specific growth rate of at least 0.4/hr and Saier discloses Pts- mutants having a specific growth rate of 0.35/hr, the Examiner maintains the Saier Pts- cell growth rates are very close to the instantly claimed Pts- cell growth rates, and that one of ordinary skill in the art would have reasonable expectation to be able to obtain cells within the scope of Applicants' claims. Applicants respectfully disagree with the Examiner.

Saier does not teach or suggest that a specific growth rate greater than 0.35 could be obtained. In Table 1 of the reference, four Pts- mutants are disclosed. The generation time of these mutants range from 7 hours to 2 hours. As previously acknowledged, the 2-hour generation time is equivalent to a specific growth rate of 0.35/hr. There is no teaching in Saier that a greater specific growth rate could be obtained. In general, while one may be motivated to obtain cells with a specific growth rate greater than 0.35/hr, there is no teaching or suggestion provided in the Saier reference. Additionally, there is no teaching in Saier on how to obtain Pts- mutant cells with a specific growth rate of at least 0.4/hr. Not only is the requisite suggestion lacking in the reference but also the reference lacks any suggestion on the

likelihood of success in obtaining Pts- mutant cells having a specific growth rate on glucose as a sole carbon source of at least 0.4/hr.

Applicants contend that a specific growth rate difference of 0.05/hr in microorganisms is not an insignificant difference. In the instant case, a specific growth rate difference of 0.05/hr can result in a significant decrease in generation time (min) of about 14.6% and a significant increase in glucose consumption (μm/mg/min) of about 12.6%.

The Examiner is also referred to *Escherichia coli and Salmonella*, 2nd ED, (1996) ed. F. C. Neidhardt, Chapter 97, Bremer and Dennis, "Modulation of Chemical Compositions and Other Parameters of the Cell by Growth Rate" which is attached hereto. Small changes in growth rate may result in exponential changes on the composition of cells. The Examiner is referred to Table 3 of the reference. When the growth rate (μ) changes from 0.6 to 1.0 , a number of parameters significantly change, for example, the number of ribosomes per cell doubled and the rate of mRNA synthesis per cell increased more than 2 times. These two changes will have an important affect on the ability of the cells to overproduce biomolecules such as proteins or metabolic intermediates.

Significantly, to obtain glu+ mutants the Saier Pts- parental strains were plated on minimal glucose agar medium and mutagenized with nitrosoguanidine. Applicants claimed method for obtaining Pts-/glu+ mutant strains (claim 38) teaches a different method. The Pts-/glu+ mutant cells are obtained by culturing Pts- cells under continuous culture conditions. The Pts- host cells spontaneously revert to the glu+ phenotype. Applicants contend Saier does not disclose or suggest the claimed invention. Saier neither provides motivation to one of skill in the art to produce the claimed mutant cells nor provides any expectation of successfully producing such cells.

Further, the Examiner has rejected the claims as unpatentable over Ingrahm. Ingrahm describes an alternative pathway for glucose transport, i.e. by uncoupling glucose transport into the cell from PEP utilization by incorporating the heterologous genes of GLF and hexokinase, into the Pts- host cell. See Ingrahm col.5, lines 10-19. Applicants fail to find any suggestion whatsoever in Ingrahm regarding culturing a Pts-/glu- phenotype in a media having glucose as the sole carbon source or the use of spontaneously reverted glucose+ phenotypes wherein the reversion results from culturing a Pts-/glu- phenotype in a media having glucose as the sole carbon source.

Ingrahm is concerned with a host cell mutant defective in the PTS system, see col. 5, lines 10-26, but fails to suggest or appreciate the ability to utilize native *E. coli* genes to

generate a glucose+ phenotype. While one of skill in the art reading Ingrahm would be motivated to engineer a host cell to comprise GLF and hexokinase, there is no motivation provided in the reference to obtain the claimed invention.

It appears the Examiner has rejected the above claims over Saier and Ingrahm individually. However, even if the references were combined, Applicants contend the combination would fail to render the claimed invention obvious. Saier teaches a Pts-/Glu+ phenotype wherein the glu+ phenotype was obtained by chemical mutagenesis and the highest specific growth rate obtained by the mutants was 0.35/hr. Ingrahm teaches the incorporation of heterologous genes to uncouple glucose transport in the cell from PEP utilization.

The Examiner has also rejected claims 23 – 31, 33 – 38, 41, 42, 44, 45 and 46 as being unpatentable over the combined disclosures of Frost, Holms, Ingrahm and Saier. Ingrahm and Saier are discussed above and the discussion is incorporated herein.

Frost relates to 1) increasing the amount of substrate E4P for the first committed step of the common aromatic pathway and 2) increasing expression of transketolase in the common aromatic pathway relative to a wild type which comprises incorporation of a recombinant *tkt* gene in the host cell. There is no teaching or suggestion in Frost to obtain a Pts-/Glu+ phenotype by inactivating or deleting a gene selected from the group of *ptsI*, *ptsH* and *crr*.

Holms provide a general discussion related to central metabolic pathways of *E. coli* and discusses the role of PEP in those pathways. While Holms may disclose that the PTS system consumes 66% of the PEP produced while only 3% of the PEP pool is channeled into aromatic amino acid synthesis, the reference has no teaching or suggestion regarding the type of pathway engineering associated with increasing PEP availability in a host cell as claimed by Applicants.

Applicants submit the references applied individually clearly do not support the Examiner's rejection and moreover a combination of the references in any manner also fails to support the Examiner's *prima facie* case of obviousness.

Frost does not teach a method of increasing PEP availability or a cell having a Pts-/glu+ phenotype wherein the phenotype is obtained by the deletion or inactivation of one or more genes selected from *ptsI*, *ptsH* and *crr*. Additionally Frost does not teach or suggest a method of making a Pts-/glu+ including culturing a mutant cell having a Pts-/glu+ phenotype under continuous culture conditions.

Holms merely teaches that PEP within *E. coli* is consumed by several different metabolic pathways and the amount of PEP channeled into each pathway. Saier teaches mutagenized cells with a Pts-/glu+ phenotype having a growth rate of 0.35, but there is no suggestion of

obtaining Pts-/glu+ cells with a growth rate of at least 0.4/hr. Ingrahm discusses a host cell mutant defective in the PTS system but fails to suggest or appreciate the ability to utilize native *E. coli* genes to generate a glucose+ phenotype.

Even if the references are combined, Applicants contend the claimed invention, as a whole is not suggested by the references. Applicants submit that the Examiner has failed to support a *prima facie* case of obviousness.

Accordingly in view of the above remarks, it is submitted that the pending claims are in form for allowance and allowance is kindly solicited.

Date: December 13, 2002

Respectfully submitted,

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Enclosures: Appendix I - Marked-up version of the amended claims
Appendix II - Clean claim set
Article by Bremer and Dennis "Modulation of Chemical Compositions and Other Parameters of the cell by Growth Rate" in Escherichia coli and Salmonella.

APPENDIX I – MARKED-UP VERSION OF THE AMENDED CLAIMS

23. (Amended) A mutant host cell having a metabolic pathway which uses PEP as a precursor or intermediate of metabolism, said host cell characterized by:

(a) being phenotypically Pts-/glu+ **wherein the Pts- phenotype is caused by the deletion or inactivation of all or substantially all of a gene selected from the group consisting of *ptsI*, *ptsH* and *crr*;**

(b) requiring galactose permease activity to transport glucose; and

(c) having a specific growth rate on glucose as a sole carbon source of at least $0.4h^{-1}$.

27. (Amended) A method for increasing PEP availability to a biosynthetic or metabolic pathway of a host cell, the method comprising,

[culturing] a) obtaining a host cell mutant characterized by**[:]** having a Pts-/glu+ phenotype**[;]** requiring galactose permease activity to transport glucose; and having a specific growth rate on glucose as a sole carbon source of at least $0.4h^{-1}$ **wherein the Pts- phenotype is caused by the deletion or inactivation of all or substantially all of one of the genes selected from the group consisting of *ptsI*, *ptsH* and *crr*; and**

b) culturing the host cell mutant in the presence of an appropriate carbon source, wherein said host cell mutant utilizes PEP as a precursor or intermediate of metabolism.

29. (Amended) **[A] The** method of Claim 27 further comprising modifying the **[selected]** host cell **mutant** to introduce therein recombinant DNA coding one or more of the enzymes selected from the group consisting of transketolase, transaldolase and phosphoenolpyruvate synthase such that the mutant host cell expresses transketolase, transaldolase or phosphoenolpyruvate synthase at enhanced levels relative to wild-type host cells.

30.(Amended) **[A] The** method of Claim 27 further comprising modifying the **[selected]** host cell **mutant** to reduce or eliminate pyruvate kinase activity in said host cell.

38. (Amended) A method for obtaining a Pts- /Glucose$^+$, galactose permease requiring-mutant cell, the method comprising:

(a) selecting a host cell which utilizes a phosphotransferase transport system;

(b) mutating the host cell whereby the phosphotransferase transport system is inactivated;

(c) culturing the mutant host cell **under continuous culture** conditions using glucose as a carbon source; and

(d) selecting mutant host cells which grow on glucose at a specific growth rate of at least $0.4h^{-1}$.

39. (Amended) **[A method of Claim 38 wherein the] A method for obtaining a Pts-/Glucose$^+$, galactose permease requiring-mutant cell, the method comprising:**

(a) selecting a host cell which utilizes a phosphotransferase transport system;

(b) mutating the host cell whereby the phosphotransferase transport system is inactivated;

(c) culturing the mutant host cell using glucose as a carbon source; and

(d) selecting mutant **host** cells **[are selected due to] having** a specific growth rate on glucose of about $0.8h^{-1}$.

40. (Amended) **[The mutant cell of Claim 23] A mutant host cell having a metabolic pathway which uses PEP as a precursor or intermediate of metabolism, said host cell characterized by:**

(a) being phenotypically Pts-/Glu$^+$;

(b) requiring galactose permease activity to transport glucose; and

(c) having a specific growth rate on glucose as a sole carbon source of about $0.8h^{-1}$.

42. (Amended) A method for enhancing production of a desired compound in a modified host cell, said host cell in its unmodified form being capable of utilizing a phosphotransferase transport system for carbohydrate transport, the method comprising,

(a) **[culturing a modified host cell with an appropriate carbon source,] obtaining a modified host cell, wherein** said modified host cell **is** characterized by having

(i) a Pts-/glu+ phenotype;

(ii) requiring galactose permease activity to transport glucose;

(iii) having a specific growth rate on glucose as a sole carbon source of at least about $0.4h^{-1}$; and

(iv) utilizing PEP as a precursor or intermediate of metabolism,

said modified host cell further comprising recombinant DNA encoding one or more enzyme(s) catalyzing reactions in the pathway of biosynthetic production of said desired compound in said modified host cell; and

(b) **[optionally recovering said compound]** **culturing the modified host cell with an appropriate carbon source whereby the production of a desired compound in the modified host cell is enhanced compared to the production of said desired compound in the unmodified host cell**.

43. (Amended) **[The method of Claim 42, wherein the modified host cell has]** **A method for enhancing production of a desired compound in a modified host cell, said host cell in its unmodified form being capable of utilizing a phosphotransferase transport system for carbohydrate transport, the method comprising,**

(a) obtaining a modified host cell, said modified host cell characterized by having

(j) a Pts-/glu+ phenotype;

(ii) requiring galactose permease activity to transport glucose;

(iii) a specific growth rate on glucose as a sole carbon source of about $0.8h^{-1}$ **and**

(iv) utilizing PEP as a precursor or intermediate of metabolism, said modified host cell further comprising recombinant DNA encoding one or more enzymes catalyzing reactions in the pathway of biosynthetic production of said desired compound in said modified host cell and

(b) culturing the modified host cell with an appropriate carbon source whereby the production of a desired compound in the modified host cell is enhanced compared to the production of said desired compound in the unmodified host cell.

APPENDIX II- PENDING CLAIM SET

23. (Amended) A mutant host cell having a metabolic pathway which uses PEP as a precursor or intermediate of metabolism, said host cell characterized by:

(a) being phenotypically Pts-/glu+ wherein the Pts- phenotype is caused by the deletion or inactivation of all or substantially all of a gene selected from the group consisting of *ptsI*, *ptsH* and *crr*;

(b) requiring galactose permease activity to transport glucose; and

(c) having a specific growth rate on glucose as a sole carbon source of at least $0.4h^{-1}$.

24. (Reiterated) A mutant host cell of Claim 23 comprising recombinant DNA coding for one or more of the enzymes selected from the group consisting of transketolase, transaldolase and phosphoenolpyruvate synthase such that the mutant host cell expresses transketolase, transaldolase or phosphoenolpyruvate synthase at enhanced levels relative to wild-type host cells.

25. (Reiterated) A mutant host cell of Claim 23 further comprising mutations in the pykA and/or pykF genes in said host cell.

26. (Reiterated) A mutant host cell of Claim 24 further comprising mutations in the pykA and/or pykF genes in said host cell.

27. (Amended) A method for increasing PEP availability to a biosynthetic or metabolic pathway of a host cell, the method comprising,

a) obtaining a host cell mutant characterized by having a Pts-/glu+ phenotype requiring galactose permease activity to transport glucose; and having a specific growth rate on glucose as a sole carbon source of at least $0.4h^{-1}$ wherein the Pts- phenotype is caused by the deletion or inactivation of all or substantially all of one of the genes selected from the group consisting of *ptsI*, *ptsH* and *crr*; and

b) culturing the host cell mutant in the presence of an appropriate carbon source, wherein said host cell mutant utilizes PEP as a precursor or intermediate of metabolism.

28. (Canceled)

29. (Amended) A method of Claim 27 further comprising modifying the host cell mutant to introduce therein recombinant DNA coding one or more of the enzymes selected from the group consisting of transketolase, transaldolase and phosphoenolpyruvate synthase such that the mutant host cell expresses transketolase, transaldolase or phosphoenolpyruvate synthase at enhanced levels relative to wild-type host cells.

30. (Amended) The method of Claim 27 further comprising modifying the host cell mutant to reduce or eliminate pyruvate kinase activity in said host cell.

31. (Reiterated) A method of Claim 30 wherein pyruvate kinase activity is reduced or eliminated in the host cell by introducing a mutation in DNA encoding one or more of the sequences coding for pyruvate kinase, pyruvate kinase promoter region and other regulatory sequences controlling expression of pyruvate kinase.

33. (Reiterated) A method of Claim 42 wherein the DNA used to transform the host cell encodes one or more enzyme(s) selected from the group consisting of DAHP synthase, DHQ synthase, DHQ dehydratase, shikimate dehydrogenase, shikimate kinase, EPSP synthase and chorismate synthase.

34. (Reiterated) A method of Claim 42 further comprising transforming the host cell with recombinant DNA coding one or more enzyme(s) selected from the group consisting of transketolase, transaldolase and phosphoenolpyruvate synthase so that said enzyme is expressed at enhanced levels relative to wild-type host cells.

35. (Reiterated) A method of Claim 33 further comprising transforming the host cell with recombinant DNA coding one or more enzyme(s) selected from the group consisting of transketolase, transaldolase and phosphoenolpyruvate synthase so that said enzyme is expressed at enhanced levels relative to wild-type host cells.

36. (Reiterated) A method of Claim 42 wherein the desired compound is selected from the group consisting of tryptophan, tyrosine and phenylalanine.

37. (Reiterated) A method of Claim 36 wherein the desired compound is tryptophan and the host cell is transformed with DNA coding one or more gene(s) selected from the group consisting of *aroG, aroA, aroC, aroB, aroL, aroE, trpE, trpD, trpC, trpB, trpA* and *tktA* or *tktB*.

38. (Amended) A method for obtaining a Pts- /Glucose$^+$, galactose permease requiring-mutant cell, the method comprising:

(a) selecting a host cell which utilizes a phosphotransferase transport system;

(b) mutating the host cell whereby the phosphotransferase transport system is inactivated;

(c) culturing the mutant host cell under continuous culture conditions using glucose as a carbon source; and

(d) selecting mutant host cells which grow on glucose at a specific growth rate of at least $0.4h^{-1}$.

39. (Amended) A method for obtaining a Pts- /Glucose$^+$, galactose permease requiring-mutant cell, the method comprising:

(a) selecting a host cell which utilizes a phosphotransferase transport system;

(b) mutating the host cell whereby the phosphotransferase transport system is inactivated;

(c) culturing the mutant host cell using glucose as a carbon source; and

(d) selecting mutant host cells having a specific growth rate on glucose of about $0.8h^{-1}$.

40. (Amended) A mutant host cell having a metabolic pathway which uses PEP as a precursor or intermediate of metabolism, said host cell characterized by:

(a) being phenotypically Pts- /Glu$^+$;

(b) requiring galactose permease activity to transport glucose; and

(c) having a specific growth rate on glucose as a sole carbon source of about $0.8h^{-1}$.

41. (Canceled)

42. (Amended) A method for enhancing production of a desired compound in a modified host cell, said host cell in its unmodified form being capable of utilizing a phosphotransferase transport system for carbohydrate transport, the method comprising,

(a) obtaining a modified host cell, wherein said modified host cell is characterized by having

(i) a Pts-/glu+ phenotype;

(ii) requiring galactose permease activity to transport glucose;

(iii) having a specific growth rate on glucose as a sole carbon source of at least about $0.4h^{-1}$; and

(iv) utilizing PEP as a precursor or intermediate of metabolism,

said modified host cell further comprising recombinant DNA encoding one or more enzyme(s) catalyzing reactions in the pathway of biosynthetic production of said desired compound in said modified host cell; and

(b) culturing the modified host cell with an appropriate carbon source whereby the production of a desired compound in the modified host cell is enhanced compared to the production of said desired compound in the unmodified host cell.

43. (Amended) A method for enhancing production of a desired compound in a modified host cell, said host cell in its unmodified form being capable of utilizing a phosphotransferase transport system for carbohydrate transport, the method comprising,

(a) obtaining a modified host cell, said modified host cell characterized by having

(k) a Pts-/glu+ phenotype;

(ii) requiring galactose permease activity to transport glucose;

(iii) a specific growth rate on glucose as a sole carbon source of about $0.8h^{-1}$ and

(iv) utilizing PEP as a precursor or intermediate of metabolism, said modified host cell further comprising recombinant DNA encoding one or more enzymes catalyzing reactions in the pathway of biosynthetic production of said desired compound in said modified host cell and

(b) culturing the modified host cell with an appropriate carbon source whereby the production of a desired compound in the modified host cell is enhanced compared to the production of said desired compound in the unmodified host cell.

44. (Reiterated) The method of Claim 42 wherein the Pts- phenotype is caused by the deletion or inactivation of all or substantially all of one or more gene(s) selected from the group consisting of ptsI, ptsH and crr.

45. (Reiterated) The method of Claim 38 wherein mutating the host cell is by inactivating the phosphotransferase transport system.

46. (Reiterated) The method of Claim 45 wherein said inactivating is by deleting part or all of gene(s) selected from the group consisting of ptsI, ptsH and crr.

47. (New) The mutant host cell of claim 40 further comprising mutations in a gene selected from the group *pykA* and *pykF*.

48. (New) The mutant host cell of claim 40 further comprising recombinant DNA coding for one or more of the enzymes selected from the group consisting of transketolase, transaldolase, and phosphoenolpyruvate synthase wherein the mutant host cell expresses transketolase, transaldolase or phosphoenolpyruvate synthase at enhanced levels relative to wild-type host cells.

49. (New) The method of Claim 38, wherein the selected mutant host cell has a specific growth rate of at least 50% of the host cell of step a).

50. (New) The method of Claim 42 further comprising recovering said desired compound.

51. (New) The method of Claim 43 further comprising recovering said desired compound.

Escherichia coli and *Salmonella*

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Modulation of Chemical Composition and Other Parameters of the Cell by Growth Rate

HANS BREMER AND PATRICK P. DENNIS

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HISTORY

Schaechter et al. (121) first demonstrated that the macromolecular composition of the bacterial cell was related to its metabolic activity and that RNA-containing particles were involved in the synthesis of protein. When they examined the variations in growth and composition of *Salmonella typhimurium* cultures in different media, they realized that the cellular contents of DNA, RNA, and protein at a given temperature depended only on the growth rate and not on the nutrient supplement in the growth medium used to achieve that growth rate. They also found (i) that fast-growing bacteria are larger and contain more DNA, RNA, and protein than slow-growing bacteria, (ii) that the amounts of these macromolecules are exponential functions of growth rate, and (iii) that the exponents of these functions are different for different macromolecules. The last implies that the relative proportions of the different macromolecules change with growth rate; at a given temperature, RNA and ribosome concentration increase with increasing growth rate, DNA concentration decreases, and protein concentration remains almost constant. When the growth rate was varied by changing the temperature rather than the nutrient content of the growth medium, the DNA, RNA, and protein concentrations remained invariant.

These early studies of bacterial physiology are documented by Maaløe and Kjeldgaard (95). The statement of observations in terms of simple mathematical relationships was characteristic for the "Copenhagen approach," in which calculated constants, proportionalities, and quadratic or exponential functions suggested special control mechanisms. Many of these relationships later turned out to be more complex than originally imagined. Nevertheless, these propositions have stimulated thought and led to more sophisticated observations; the book continues to be provocative, as many of the fundamental problems posed in it are still far from being solved.

A theoretical basis for explaining the empirical relationships of the early Copenhagen school was not available until Cooper and Helmstetter (32) derived a formula for determining the average amount of DNA per cell in an exponential culture as a function of C (the time required to replicate the chromosome), D (the time period between termination of a round of replication and the following cell division), and τ (the culture doubling time). This theory also included the important concept of overlapping rounds of chromosome replication, where a round of replication is initiated before the previous round is completed. This occurs when τ is less than C and explains how bacteria are able to grow with a doubling time shorter than the time required for chromosome replication.

Donachie (51) extended this theory by introducing the concept of the "initiation mass" (the cell mass per replication origin at the time of initiation) and derived the average mass per cell, or amount of protein per cell, as a function of C, D, τ, and an additional parameter, mass or protein per replication origin (M_O or P_O, respectively). The Cooper-Helmstetter equation predicts the amount of DNA per cell as a function of C, D, and τ; the additional parameter, P_O, links the amount of protein to the amount of DNA.

At about the same time, Schleif (122) and Maaløe (93) began to establish a theoretical relationship between the amounts of protein and RNA in the cell and the two parameters, c_p and β_r, which define ribosome function (β_r is the fraction of total ribosomes actively engaged in peptide chain elongation; c_p is the rate of peptide chain elongation). Based on these relationships, Churchward et al. (27) were able to describe the global cell composition in terms of DNA, RNA, and protein content as a function of the doubling time, τ, and five additional parameters, C, D, P_O, β_r, and c_p.

(It is noteworthy that these parameters include the peptide and DNA chain elongation rates [C period], but not the RNA chain growth rate. Intuitively it would seem that all three chain elongation rates should be equally important and contribute to the DNA, RNA, and protein content. The explanation for this paradox is that the RNA chain elongation rate is implicit in the value of τ; if one asks for the composition as a function of growth rate, one assumes τ as a given parameter without asking how its value was achieved. Thus the cell composition is indeed determined by all three macromolecular chain elongation rates.)

TABLE 1 Parameters related to the growth and macromolecular composition of bacterial cells

Class	No.	Parameter	Symbol	Value	Reference
I	1	Deoxyribonucleotide residues per genome	kbp/genome	4,700	4
	2	Ribonucleotide residues per rRNA precursor	nucl./prib	6,000	104
	3	Ribonucleotide residues per 70S ribosome	nucl./rib	4,566	104
	4	Amino acid residues per 70S ribosome	aa/rib	7,336	140
	5	Ribonucleotide residues per tRNA	nucl./tRNA	80	64
	6	Amino acid residues per RNA polymerase core	aa/pol	3,407	107–109
II	7	Fraction of total RNA that is stable RNA	f_s	0.98	5, 80
	8	Fraction of stable RNA that is tRNA	f_t	0.14	37, 118
	9	Fraction of active ribosomes	β_r	0.8	57
III	10	Fraction of total protein that is r-protein	α_r	0.09–0.22	Table 3
	11	Fraction of total protein that is RNA polymerase	α_p	0.009–0.01	Table 3
	12	Fraction of active RNA polymerase synthesizing rRNA and tRNA	ψ_s	0.28–0.77	Table 3
	13	Fraction of active RNA polymerase	β_p	0.15–0.32	Table 3
IV	14	Peptide chain elongation rate	c_p	12–22 aa/s	Table 3
	15	Stable RNA chain elongation rate	c_s	85 nucl./s	Table 3
	16	mRNA chain elongation rate	c_m	40–55 nucl./s	Table 3
	17	DNA chain elongation rate	c_d	500–830 nucl. bp/s	Table 3
V	18	Time to replicate the chromosome	C	40–67 min	Table 3
	19	Time between termination of replication and division	D	22–30 min	Table 3
	20	Protein per replication origin	P_O	2.5×10^8–4×10^8 aa	Table 2

OBSERVED CELL COMPOSITION OF *E. COLI* B/r

Cell Growth-Related Parameters

In Table 1, a number of growth-related parameters are listed that are generally useful in describing or establishing the macromolecular composition of bacterial cultures. These parameters can be divided into five classes: (i) structural parameters that are inherently constant and do not vary with the growth rate, like the number of rRNA nucleotides in a 70S ribosome; (ii) partition factors which are essentially invariant and growth rate independent, like the fraction of total RNA that is stable rRNA; (iii) other partition parameters which change as a function of the exponential growth rate and have substantial effects on cell composition, like the fraction of active RNA polymerase synthesizing rRNA and tRNA; (iv) kinetic parameters describing functional activities (the values of some of the parameters are essentially invariant, whereas others appear to approach a maximum or biological limit value, like the peptide chain elongation rate); (v) chromosome replication and cell division parameters that in general do not limit the exponential growth rate, like the C period.

Reference Units

Physiological parameters describing cell composition, like the amount or synthesis rate of a particular component, require a reference unit such as "per cell," "per cell mass," "per amount of protein," "per microgram of dry weight," etc. Except for studies dealing with the cell cycle, we recommend in most cases the use of cell mass (e.g., "per OD_{460}" [unit of optical density at 460 nm]) as a reference unit because its determination is simpler, faster, and more accurate than that of other units. The per-mass values may also be used to estimate the intracellular concentrations because the average cell volume per mass unit changes very little with growth conditions. In many cases the concentration is more relevant than the amounts of components per genome equivalent of DNA or per cell. Since the reference parameters are themselves

subject to growth rate-dependent regulation, no single reference unit is ideal or more natural than another.

There has been a tendency in the recent literature for authors to state that rRNA or ribosomes accumulate in proportion to the square of the growth rate. This, of course, is incorrect because the unit of reference is undefined. As first pointed out by Maaløe (93), the rate of RNA accumulation per genome does increase with the square of the growth rate, μ. This reflects the fact that the amount of RNA per genome is proportional to the growth rate (at least above growth rates of 0.5 doubling per h). Since in any exponential system the synthesis rate is proportional to the amount, and the amount is already proportional to μ, it follows that the rate must be proportional to μ^2. However, since the ratio RNA/DNA reflects both chromosome replication and RNA synthesis control, this square relationship has no particular significance for ribosome control itself; the relationship no longer holds in certain replication control mutants which alter DNA content but show no change in rRNA control (27, 28).

Macromolecular Composition as a Function of Growth Rate

For physiological studies, *Escherichia coli* B/r has several advantages over other *E. coli* strains. Due to a special property of its outer membrane, this strain can be age-fractionated by the membrane elution technique (71) and used to measure cell cycle-related parameters. Helmstetter and Cooper (70) used this strain to measure the *C* and *D* periods and deduced from these measurements the relationships between chromosome replication and the cell division cycle. This strain also (i) has a lesser tendency for clumping and "snake" formation than other strains of *E. coli,* (ii) grows well in minimal media, and (iii) is free of mutations which might otherwise influence the growth rate or composition. A disadvantage is that the strain is genetically incompatible with K-12 strains because of the B and K restriction systems. Mutants deficient in B restriction or with K-12 restriction and modification are available from the authors. For these reasons, *E. coli* B/r is the preferred choice for physiological studies and composition measurements.

Table 2 lists the amounts of protein, RNA, and DNA and related physiological parameters for cultures of *E. coli* B/r growing exponentially at 37°C in different growth media at rates between 0.6 and 2.5 doublings per h. The per-mass values (top section) represent averages obtained from curves drawn as a best fit through individually measured points (28). The actual measurements fluctuate by about 15% around these curves. Most of this scatter represents a true variation from culture to culture (the contribution due to measuring errors is about ±6%, 2.5%, 5%, and 5% for protein, RNA, DNA, and cell number, respectively). Protein, RNA, and DNA were measured colorimetrically, and cell numbers were determined with an electronic particle counter as indicated in Table 2. From the per-mass values, protein and RNA per genome and protein, RNA, and DNA per cell were calculated.

The sums of the weights of protein, RNA, and DNA are proportional to the mass in OD_{460} units and correspond to 75 to 91% of the dry weight; lipids, carbohydrates, soluble metabolites, and salts represent the remaining 9 to 25% of the total dry mass. The relative proportions of the macromolecules at the different growth rates are illustrated in the bar graphs of Fig. 1. The greatest relative change is found in the RNA sector, reflecting the increasing concentration of ribosomes at higher growth rates. More ribosomes are required to support the higher rate of protein synthesis in rapidly growing cells.

TABLE 2 Macromolecular composition of exponentially growing *E. coli* B/r as a function of growth rate at 37°C[a]

Parameter	Symbol	Units	At τ (min) and μ (doublings per h): τ, 100 μ, 0.6	τ, 60 μ, 1.0	τ, 40 μ, 1.5	τ, 30 μ, 2.0	τ, 24 μ, 2.5	Observed parameter(s)	Footnote
Protein/mass	P_M	10^{17} aa/OD_{460}	6.5	5.8	5.2	5.1	5.0	*P, M*	*b*
RNA/mass	R_M	10^{16} nucl./OD_{460}	4.3	4.9	5.7	6.6	7.8	*R, M*	*c*
DNA/mass	G_M	10^8 genomes/OD_{460}	18.3	12.4	9.3	8.0	7.6	*G, M*	*d*
Cell no./mass	C_M	10^8 cells/OD_{460}	11.7	6.7	4.0	2.7	2.0	Cells/OD_{460}	*e*
$(P + R + G)/M$	PRD_M	μg/OD_{460}	149	137	129	131	136		*f*
Protein/genome	P_G	10^9 aa residues	3.5	4.7	5.6	6.3	6.6	P_M, G_M	
RNA/genome	R_G	10^7 nucl. residues	2.3	4.0	6.1	8.2	10.3	R_M, G_M	
Origins/genome	O_G	Dimensionless	1.25	1.32	1.44	1.58	1.73	*C*	*g*
Protein/origin	P_O	10^8 aa residues	2.8	3.6	3.9	4.0	3.8	P_G, O_G	*g*
Protein/cell	P_C	10^9 aa residues	5.6	8.7	13.0	18.9	25.0	P_M, C_M	
	P_C (μg)	μg/10^9 cells	100	156	234	340	450		*h*
RNA/cell	R_C	10^7 nucl. residues	3.7	7.3	14.3	24.4	39.0	R_M, C_M	
	R_C (μg)	μg/10^9 cells	20	39	77	132	211		*h*
DNA/cell	G_C	genome equiv./cell	1.6	1.8	2.3	3.0	3.8	*C, D*	*i*
	G_C (μg)	μg/10^9 cells	7.6	9.0	11.3	14.4	18.3		*h*
Mass/cell	M_C	OD_{460} units/10^9cells	0.85	1.49	2.5	3.7	5.0	C_M	*j*
	M_C (μg)	μg dry weight/10^9 cells	148	258	433	641	865	μg/OD_{460}	*k*
Sum $P + R + G$	PRD_C	μg/10^9 cells	127	204	322	486	679	P_C, R_C, G_C (in μg)	*k*
Origins/cell	O_C	no./cell	1.96	2.43	3.36	4.70	6.54	*C, D*	*l*
Termini/cell	T_C	no./cell	1.23	1.37	1.54	1.74	1.94	*D*	*l*
Replication forks/cell	F_C	no./cell	1.46	2.14	3.64	5.92	9.19	*C, D*	*l*

[a]Data are representative for the growth rates indicated, with an accuracy of ±10% or better. In compiling the data in this table and Table 3, we have been guided by the principle that, on average, parameters like protein, RNA, DNA, and cell number per mass, and their quotients, i.e., protein and RNA per genome, or the per-cell values, should have smooth functions of growth rate. (If two primary data were 5% off the true average, their quotient might contain a 10% error and thus make it impossible to draw a smooth line through the points.) In addition, we have checked for consistency if measurements were available from independent methods or involved theoretical relationships between different parameters. The data in our tables closely meet these criteria. For example, RNA, measured as absorption at 260 nm (A_{260}) of RNA hydrolysates, does not require a calibration standard; therefore, the RNA values are assumed to be quite accurate. Since the RNA-to-protein ratios from this table generated the same α_r curve as that determined from purified ribosomal particles (Table 3), we have confidence in both RNA and protein values. The amount of DNA per mass was measured independently with a colorimetric assay calibrated with purified *E. coli* DNA and by radioactive pyrimidine labeling of nucleic acids. The latter method gives the RNA-to-DNA nucleotide ratio which, combined with the absolute (presumably reliable) value for RNA per mass, gives DNA per mass (45). Again both methods gave essentially the same values. Thus, all three macromolecular concentrations (per mass) in this table have consistent values which are presumably accurate to better than 10% and representative for that growth rate. Representative per-cell values were more difficult to obtain, in part because the duration of the (average) *D* period, which affects the cell size and per-cell values, fluctuates considerably from culture to culture; this fluctuation is independent of and in addition to the variation in *D* from cell to cell within one culture (24). The DNA per cell values have been determined directly from DNA and cell numbers per mass and indirectly from the *C* and *D* periods. Both methods gave essentially the same DNA content of the average cell. Abbreviations: aa, amino acid; nucl., nucleotide; equiv., equivalent.

[b]Cell mass density was determined as OD_{460} using a 1-cm light path (27). Protein was determined by a modification of the method of Lowry et al. (20, 92), using (weighted) bovine serum albumin as a calibration standard and assuming 5.6×10^{15} amino acid residues per μg of protein. The values shown are from Fig. 3 of Churchward et al. (29).

[c]RNA was determined as A_{260} of acid-insoluble, alkali-labile cell mass (20). One A_{260} unit at pH 2 corresponds to 5.6×10^{16} nucleotides, assuming the mole fractions of A, U, G, and C in *E. coli* stable RNA to be 0.248, 0.210, 0.324, and 0.218, respectively (102). The RNA values shown are from Churchward et al. (28).

[d]DNA per mass was calculated from DNA per cell and cells per mass: $G_M = G_C \cdot C_M$. These calculated values closely agree with direct measurements of DNA per mass (Fig. 1 of reference 29), using the colorimetric diphenylamine reaction with *E. coli* DNA as a calibration standard and assuming 1 A_{260} unit of *E. coli* DNA at pH 12 to correspond to 2.86×10^{13} kbp (for a GC content of 0.50) and the *E. coli* genome to be 4,700 kbp (4).

[e]Cell numbers were determined using a Coulter Counter with a 20-μm orifice. The values shown are from Fig. 6 of Shepherd et al. (125).

[f]The sum of the weights of protein, RNA, and DNA per mass was calculated from the sum of the weights per cell (see footnote *h*, this Table) and cells per mass: $PRD_M = PRD_C \cdot C_M$.

[g]The number of replication origins per genome was determined from the value of the *C* period (Table 3), using equation 11 in Table 5 below.

[h]The weights of protein, RNA, and DNA were calculated, assuming the average molecular weight of an amino acid residue in *E. coli* protein to be 108 (composition of *E. coli* protein from reference 133), that of an RNA nucleotide residue to be 324 (composition of *E. coli* stable RNA from reference 102), and that of a DNA base pair to be 618 (for a GC content of 0.50), respectively.

[i]The average amount of DNA per cell, in genome equivalents, was calculated from the values of *C* and *D* (Table 3), using equation 3 in Table 5 below. These calculated values agree with direct (colorimetric) measurements of DNA and cell numbers per mass (reference 28; see footnote *d* in this Table).

[j]The average cell mass in OD_{460} units is the reciprocal of the cell number per OD_{460}, i.e., $M_C = 1/C_M$.

[k]The cell mass in micrograms dry weight was calculated, using the value of 173 μg per OD_{460} unit of culture mass (20).

[l]The average numbers of replication origins, termini, and replication forks were determined from the values of *C* and *D* (Table 3), using equations 7, 8, and 10, respectively, of Table 5 below.

The growth rate-dependent changes in the relative proportions of DNA, RNA, and protein can be described by the two ratios, RNA/protein and DNA/protein. With increasing growth rate, RNA/protein increases and DNA/protein decreases (Fig. 2a and b). The increasing RNA/protein ratio reflects the control of ribosome synthesis (see equation 18 in Table 6 below), and the decreasing DNA/protein ratio reflects the control of DNA

replication (see legend to Fig. 2j). The RNA/protein ratio is proportional to the number of ribosomes per amount of protein and, therefore, is a measure for the cytoplasmic ribosome concentration. The growth rate of an exponential culture is equal to the product of ribosome concentration times the rate of ribosome function (i.e., the protein synthesis rate per average ribosome or the ribosome efficiency; 74, 122). Therefore, at a given growth rate, the protein synthesis rate per ribosome can be calculated from the RNA/protein ratio. When the growth rate increases, the rate of ribosome function approaches a maximum value, corresponding to 21 amino acids polymerized per second per active ribosome (Fig. 2c, right ordinate scale).

The number of replication origins in a culture was obtained by measuring the amount of DNA that had accumulated 50 to 80 min after treatment of a culture with rifampin. Rifampin stops initiation of replication, but allows the ongoing rounds of replication to go to completion, so that the number of completed chromosomes becomes equal to the number of functional origins present at the time of rifampin addition (128, 129). The mass per origin, M_O, was then obtained by dividing this number of completed chromosomes by the OD_{460} observed at the time of rifampin addition. The amount of protein per origin, P_O, was found from M_O by multiplication with the amount of protein per mass, P_M (Table 2).

Protein per origin, P_O, is a formal measure for the control of replication initiation; it has a meaning similar to cell mass per origin, M_O, which is proportional to the "initiation mass" defined by Donachie (51). The initiation mass is the cell mass at the time of initiation, divided by the number of replication origins at which initiation occurs, i.e., M_i/O_i (Fig. 2j), whereas M_O is the total mass in a given volume of exponential culture, divided by the number of copies of *oriC* present in that volume. Both P_O and M_O increase with growth rate and approach a constant value at growth rates above 1.5 doublings per h (Fig. 2j; $P_O = 4 \times 10^8$ amino acids per *oriC*). The exact growth rate dependence of P_O (or M_O) may depend on the strain used. A decreasing initiation mass with increasing growth rate has been reported for a K-12 strain of *E. coli* (130).

The parameter P_O links DNA replication to protein synthesis and growth. Whereas the time intervals between consecutive cell divisions vary considerably, the time intervals between consecutive initiations of rounds of replication vary very little (83). This is presumed to reflect the accumulation of a hypothetical protein that triggers initiation at a certain threshold value (17). This putative initiation protein would be made as a constant fraction of total protein synthesis, thereby linking chromosome replication to protein synthesis.

The numbers of replication termini and of forks on the chromosome were calculated from the values of the C and D periods (taken from Table 3, below). These numbers relate to the extent of chromosome branching as a result of increasing overlap in rounds of replication as the cells grow faster (Fig. 1).

Parameters Pertaining to the Macromolecular Synthesis Rates

The rates of accumulation of protein, RNA, and DNA or the rate of cell division (or of any other extensive property, X, of the system) can be calculated using the first-order rate equation $dX/dt = Xk\mu = X(\ln 2)/\tau$, where μ is in doublings per hour, τ is in minutes, and $k = (\ln 2)/60$; the rate is per minute.

For DNA, ribosomes, and protein, the rates of synthesis during periods of balanced growth are essentially equal to the rates of accumulation since their turnover is negligible (37, 38). For total RNA, however, the instantaneous synthesis rate is substantially higher than the accumulation rate because of the instability of mRNA and of spacer sequences in the primary rRNA and tRNA transcripts.

In Table 3, physiological parameters related to the macromolecular synthesis rates have been divided into three groups: parameters pertaining to (i) RNA polymerase synthesis and function, (ii) ribosome synthesis and function, and (iii) DNA synthesis and cell division. Some of these parameters were observed, and others were calculated as indicated (see Table 3 footnotes).

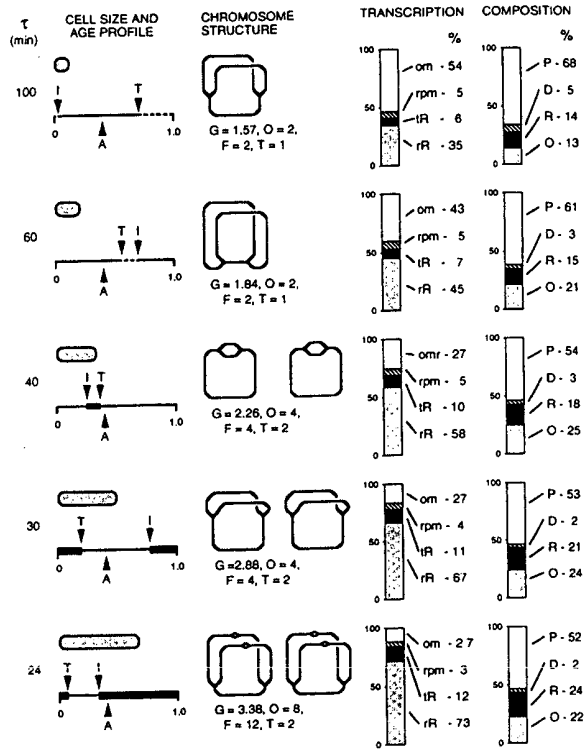

FIGURE 1 Relationships between growth rate, cell size, chromosome replication, transcription, and macromolecular composition. (Left column) Average cell size (mass per cell, Table 2) for *E. coli* B/r growing with a doubling time, τ, ranging from 100 to 24 min is depicted by the shaded ovals. An idealized cell cycle with the major cell cycle events, ranging from cell age 0.0 (a newborn daughter cell) to 1.0 (a dividing mother cell), is presented for each growth rate. The position of an average cell of age 0.41 (defined so that 50% of the cells in the population are younger and 50% are older) is indicated by A. The cell ages at initiation (I) and termination (T) of chromosome replication are also indicated. The dashed portion of the age axis indicates a period during which there is no DNA replication (no replication forks on the chromosome). The line portions represent periods where there are two forks per chromosome structure, and the heavy bar portions indicate the age periods during which there are six forks per chromosome structure. After termination, there are two chromosome structures per cell which are segregated to the daughter cells at the subsequent cell division (at age 1.0). (Center column) Structure of the replicating chromosome or chromosomes in the average cell of age 0.41. For a 24-min cell cycle (τ = 24 min), the chromosome pattern indicates that replication is being initiated and that each of these chromosome structures has multiple (six) replication forks. The amount of DNA in these structures in genome equivalents (*G*) is indicated (calculated from *C*, *D*, and τ in Table 2 for a cell of age 0.41). The number of origins (*O*), termini (*T*), and forks (*F*) in this average cell are also indicated. (Right columns) The synthesis rates of rRNA (rR), tRNA (tR), r-protein mRNA (rpm), and other mRNA (om), expressed as a percent of total transcription, and the macromolecular composition are illustrated in bar graph form. The stable RNA fraction of the total transcription increases with increasing growth rate, the r-protein mRNA fraction remains essentially constant, and the total mRNA fraction decreases. The proportion of the total mRNA that is r-protein mRNA clearly increases, approximately in parallel with the increase in α_r, the fraction of the total protein synthesis that is r-protein, implying that there is transcriptional control of r-protein operons (the transcription values are adapted from references 42 63, 89 and P. Dennis, unpublished data). Relative amounts of protein (P), DNA (D), RNA (R), and other components (O) as percent of the total cell mass are from the data in Table 2.

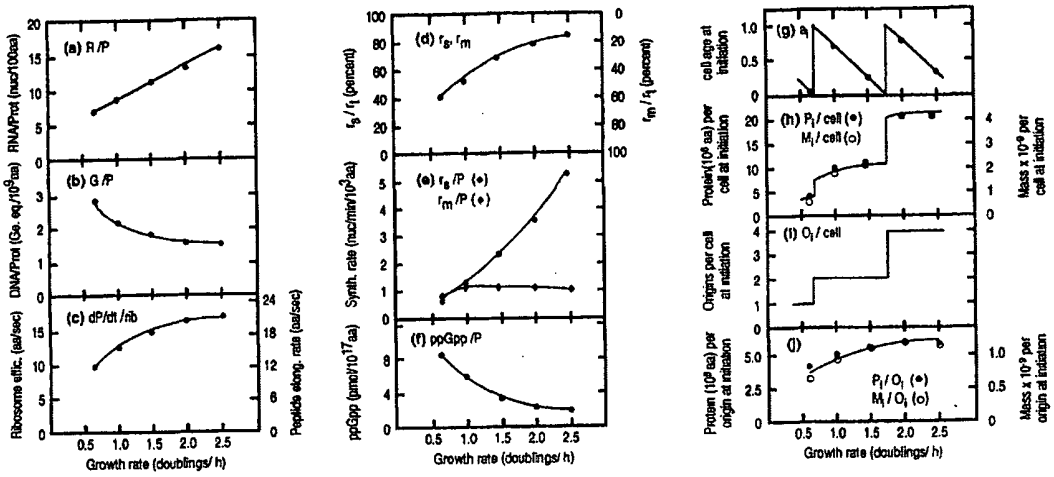

FIGURE 2 Amounts and synthesis rates of molecular components in bacteria growing exponentially at rates between 0.6 and 2.5 doublings per h. The values of the RNA-to-protein (*R/P;* panel a) and DNA-to-protein (*G/P;* panel b) ratios were calculated from lines 1, 2, and 3 in Table 2. The ribosome efficiency (i.e., the protein synthesis rate per average ribosome; panel c, left ordinate) was calculated from the number of ribosomes per cell (line 15, Table 3) and the rate of protein synthesis per cell. The latter was obtained from the amount of protein per cell (line 10, Table 2) using the first-order rate equation. The peptide chain elongation rates (panel c, right ordinate) are 1.25-fold higher than the ribosome efficiency values and account for the fact that only about 80% of the ribosomes are active at any instant. The fraction of the total RNA synthesis rate that is stable RNA or mRNA (r_s or r_m; panel d) is from line 5, Table 3. The rates of stable RNA and mRNA synthesis per amount of protein (r_s/P or r_m/P; panel e) were calculated from lines 9 and 10, Table 3, divided by the amount of protein per cell (line 10, Table 2). The ppGpp per protein value (ppGpp/*P;* panel f) is from line 11, Table 3. The cell age at which chromosome replication is initiated at *oriC* (a_i in fractions of a generation; panel g) is calculated from *C* and *D* (lines 23 and 24, Table 3) and equation 14 in Table 5. The protein (or mass) per cell at replication initiation (panel h) was calculated from the initiation age (a_i, panel g) and the cell mass immediately after cell division (age zero; i.e., $a = 0$), using equation 17 in Table 5. The latter was obtained from the average protein or mass content of cells (lines 10 or 13, respectively, Table 2), using equation 16 of Table 5. The number of replication origins at the time of replication initiation (O_i, panel i) was obtained from the values of *C* and *D* (Table 3), using equation 15 of Table 5. The initiation mass (panel j), given as protein (or mass) per replication origin at the time of replication initiation, was obtained as the quotient of the values for P_i (or M_i) and O_i shown in panels h and i.

RNA Polymerase Synthesis and Function.

RNA polymerase concentration. The instantaneous rate of transcription in the cell depends on the concentration of RNA polymerase, αp, measured as the fraction of total protein that is RNA polymerase core enzyme (three subunits, α_2, β, and β'). The values of α_p increase with the growth rate and reflect the control of the synthesis of the β and β' subunits of RNA polymerase. Since the α subunit is in excess in *E. coli* (see Table 4), the amount of core enzyme would seem to be limited by the amount of β and β' subunit polypeptides. The synthesis of these subunits is under dual transcriptional control (i) at the level of initiation at an upstream promoter and (ii) at the level of termination-antitermination at an attenuator in front of the *rpoB* gene (6, 7, 40–42, 52). Both controls are growth rate dependent, but the mechanisms mediating these controls are poorly understood. The

initiation control might involve the effector nucleotide guanosine tetraphosphate (ppGpp), whereas the read-through at the attenuator might involve an autoregulation by free or active RNA polymerase (40, 52, 96). There is also translational control of the *rpoBC* mRNA (43, 47, 48). Combining the observed α_p values with the protein per cell values of Table 2, the number of RNA polymerase molecules per cell has been calculated and was found to increase from 1,500 to 11,400 between growth rates of 0.6 and 2.5 doublings per h.

RNA polymerase activity. The total complement of RNA polymerase enzyme in the cell can be partitioned into active RNA polymerase (enzymes engaged in RNA chain elongation) and inactive RNA polymerase (DNA-bound, idling polymerase; unbound, free enzyme, ready to bind to a promoter; and truly inactive, e.g., immature enzyme). The active enzyme is determined from the rate of transcription and the RNA chain elongation rate. Due to a difference in the chain elongation rates for mRNA and rRNA (see below), the calculation contains separate components for mRNA and stable RNA. The calculation shows that only 17 to 30% of the RNA polymerase enzyme is active at any instant; this fraction, β_p, is seen to increase with growth rate (125). In ppGpp-deficient strains, up to 60% of the total RNA polymerase was found to be active; this has suggested that part of the inactive polymerase is transiently stalled at ppGpp-dependent pause sites during the synthesis of mRNA (74). Most of the remaining inactive RNA polymerase might be involved in ppGpp-independent transcriptional pausing.

Within the cell, it seems that active RNA polymerase limits the rate of transcription and that the DNA template is in excess. This was demonstrated using a mutant bacterial strain that exhibits altered DNA replication control which results in a lower DNA concentration. In spite of the lower DNA concentration there was no change in the level of transcription, implying that the total rate of transcription is regulated at the level of active RNA polymerase in the cell and not the level of available DNA template (28).

What is the state of inactive RNA polymerase within the cell? Experiments with a minicell strain indicate that most of the (core) RNA polymerase is sequestered with the DNA (N. Shepherd, Ph.D. thesis, University of Texas, Dallas, 1979). This enzyme might either be bound nonspecifically to DNA (21), or, alternatively, it might have initiated transcription but is halted at some pausing site (81, 100), perhaps associating with termination-antitermination factors (66). It remains unclear why there is such a large excess of inactive RNA polymerase, and whether and how the partition between active and inactive enzyme is maintained.

Partitioning between stable RNA and mRNA synthesis. The active RNA polymerase enzyme can be further partitioned into the fractions engaged in the synthesis of stable RNA species (rRNA, tRNA, and their spacers; ψ_s) and of mRNA ($\psi_m = 1 - \psi_s$). This partitioning is strongly correlated with, and possibly controlled by, the nucleotide effector ppGpp (119). During periods of amino acid insufficiency, ppGpp is derived from the *relA*-dependent system and elicits the well-characterized stringent response (40, 49, 69). During exponential growth at different rates, ppGpp is derived from a *relA*-independent system (3, 56, 117) that involves a product of the *spoT* gene (73, 141).

Nomura and his coworkers have suggested that free ribosomes rather than ppGpp regulate transcription of *rrn* operons (77, 105). However, when free ribosomes were allowed to accumulate by limiting the concentration of the translation initiation factor IF2, rRNA synthesis was stimulated (30), not inhibited as predicted by the free ribosome feedback hypothesis. At the same time, ppGpp levels were reduced. Thus, limitation of IF2, like treatment with chloramphenicol, induces the equivalent of the relaxed response (39, 72, 84). Furthermore, in strains unable to synthesize ppGpp due to deletions in the genes for the two separate ppGpp synthetases, ψ_s was found to be growth rate-invariant (74). This observation supports the idea that ppGpp is involved in the partitioning of RNA polymerase into stable RNA- and mRNA-synthesizing fractions. In an in vitro system with purified RNA polymerase, ppGpp has been found to preferentially inhibit rRNA synthesis (e.g., references 136 and 137), whereas attempts to find a similar effect by free ribosomes have failed (77).

TABLE 3 Parameters pertaining to the macromolecular synthesis rates in exponentially growing *E. coli* B/r as a function of growth rate at 37°C

Parameter	Symbol	Units	At τ (min) and μ (doublings per h): τ, 100 μ, 0.6	τ, 60 μ, 1.0	τ, 40 μ, 1.5	τ, 30 μ, 2.0	τ, 24 μ, 2.5	Observed parameter(s)	Footnote
RNA polymerase protein/total protein	α_p	%	0.90	1.10	1.30	1.45	1.55	α_p	*a*
RNA polymerase molecules/cell	N_p	10^3 RNAP/cell	1.5	2.8	5.0	8.0	11.4	α_p, P_C	*b*
RNA polymerase activity	β_p	%	17	20	21	24	30	r_s, r_m, c_s, c_m, N_p	*c*
Active RNA polymerase per cell	N_{ap}	RNAP/cell	205	503	992	1,929	3,298		*c*
Stable RNA synthesized per total RNA synthesized	r_s/r_t	%	41	52	68	78	85	r_s/r_t	*d*
Active RNA polymerase synthesizing stable RNA	ψ_s	%	24	36	56	68	79	r_s/r_t c_s/c_m	*e*
rRNA chain elongation	c_s	Nucl./s	85	85	85	85	85	Indirect	*f*
mRNA chain elongation	c_m	Nucl./s	39	45	50	52	55	Indirect	*g*
Rate of stable RNA synthesis/cell	r_s	10^2 nucl./min/cell	3.0	9.9	29.0	66.4	132.5	R_C	*h*
Rate of mRNA synthesis/cell	r_m	10^5 nucl./min/cell	4.3	9.2	13.7	18.7	23.4	$r_s, r_s/r_t$	*i*
ppGpp concentration	ppGpp/*M*	pmol/OD_{460}	55	38	22	15	10	ppGpp/*M*	*j*
	ppGpp/*P*	pmol/10^{17} aa	8.5	6.6	4.2	2.9	2.0	P_M	*j*
r-Protein per total protein	α_r	%	9.0	11.4	14.8	17.5	21.1	P_M, R_M	*k*
			9	11	13.5	18.0	21.6	α_r	*l*
Ribosome activity	β_r	%	80	80	80	80	80	Indirect	*m*
Peptide chain elongation	c_p	aa residues/s	12	16	18	20	21	Indirect	*n*
Ribosomes/cell	N_r	10^3 ribosomes/cell	6.8	13.5	26.3	45.1	72.0	R_C, f_s, f_t	*o*
tRNA/cell	N_t	10^3 tRNA/cell	63	125	244	419	669	N_r, f_t	*p*
rrn genes/cell	N_{rrn}	Avg no./cell	12.4	15.1	20.0	26.9	35.9	*C*, *D*	*q*
rrn genes/genome	N_{rrn}/G	Avg no./genome	7.9	8.2	8.6	9.0	9.5	*C*	*r*
Initiation rate at *rrn* gene	i_{rrn}	Initiations/min/gene	4	10	23	39	58	N_r, N_{rrn}	*s*
Distance of ribosomes on mRNA	R_m/N_r	Nucl./ribosome	79	85	65	52	41	r_m, c_m, N_r	*t*
No. of translations per mRNA	N_{trans}	Ribosomes	27	33	49	70	93	P_C, r_m	*u*
DNA chain elongation	c_d	Nucl. residues/s	585	783	870	911	933	*C*	*v*
C period	*C*	min	67	50	45	43	42	Indirect	*v*
D period	*D*	min	30	27	25	24	23	Indirect	*w*

[a] The fraction of the total cell protein that is core RNA polymerase was calculated from the β and β′ content determined by sodium dodecyl sulfate-gel electrophoresis (125).

[b] The number of core RNA polymerases per cell was calculated from α_p (this table) and values of P_C (Table 2), using aa/pol (Table 1) and the relationship $N_p = P_C \cdot \alpha_p/(\text{aa/pol})$.

[c] The fraction of active RNA polymerase was calculated from values in this table, the relationship $\beta_p = (r_s/c_s + r_m/c_m)/N_p$, and the active RNA polymerase per cell, $N_{ap} = \beta_p \cdot N_p$.

[d] The fraction of the total RNA synthesis rate that is stable RNA was determined by hybridization of pulse-labeled total RNA to an rDNA probe and correction for tRNA (119). Since the pulse-labeling time (1 min) was similar in duration to mRNA lifetimes, the r_s/r_t values shown are somewhat overestimated. This results from the underestimate of the rate of mRNA synthesis due to decay of labeled mRNA during the pulse-labeling period.

[e] The fraction of RNA polymerase synthesizing stable RNA was calculated using the relationship $\psi_s = 1/\{1 + [1/(r_s/r_t) - 1](c_s/c_m)\}$.

[f] The stable RNA (or rRNA) chain growth rate was determined from 5S rRNA or tRNA labeling after rifampin addition (99, 120, 124).

[g] The mRNA chain elongation rate was determined by analysis of pulse-labeling kinetics of RNA after size fractionation (19) and by the time lag between induction of transcription of specific mRNAs (*lacZ*, *infB*) and the appearance of specific hybridization to DNA probes from the 3′ ends of the respective genes (139).

[h] The stable RNA synthesis rate per cell was determined from the data in Tables 1 and 2 and the rate equation $r_s = R_C \cdot f_s \cdot \ln 2/t$, where the factor f_s is equal to 1.2 and corrects for the 20% of the rRNA and tRNA primary transcripts that are unstable spacer or flanking sequences.

[i] The mRNA synthesis rate per cell was determined from the data in this table and the relationship $r_m = r_s\{[1/(r_s/r_t)] - 1\}$.

[j] Measurement of ppGpp was by A_{260} after separation of nucleotides by high-pressure liquid chromatography (119). ppGpp/*P* = (ppGpp/*M*)/P_M.

[k] The differential rate of r-protein synthesis was calculated from the data in Tables 1 and 2 and the relationship for α_r (equation 6, Table 5).

[l] The differential rate of r-protein synthesis was determined from measurements of the protein content of ribosomes after labeling with radioactive leucine and uridine (44, 46).

[m] The fraction of active ribosomes was measured as ribosomes in polysomes with a correction for active 70S ribosomes (57).

[n] The peptide chain elongation rate, c_p, was calculated in amino acid residues per second per ribosome from the rate of protein synthesis (dP/dt), the number of ribosomes (N_r), and the fraction of active ribosomes (β_r), using the relationship $dP/dt = N_r \cdot \beta_r \cdot c_p$ (see also the equivalent equation 5 in Table 5; 44, 49).

[o] The number of ribosomes per cell was determined from values in Tables 1 and 2 and the relationship $N_r = R_C \cdot f_s\,(1 - f_t)/(\text{nucl./rib})$, where f_s, f_t, and nucleotides per ribosome are defined Table 1.

[p] The number of tRNAs per cell was determined from values in Tables 1 and 2 and the relationship $N_t = R_C \cdot f_s \cdot f_t/(\text{nucl./tRNA})$, where f_t and nucleotides per tRNA are defined in Table 1.

[q] The number of rRNA genes per cell was calculated from the number of *rrn* genes per genome (this table) and the number of genomes per cell (Table 2): $N_{rrn} = (N_{rrn}/G) \cdot G_C$.

[r] The number of rRNA genes per genome was determined from the value of the *C* period (footnote *v*, this table) and the locations of the seven *rrn* genes (87, 89.5, 85, 72, 90.5, 57, and 3 min, respectively), using equations 11 and 12 from Table 5 below.

[s] The transcription initiation rate at each *rrn* gene was calculated from the number of ribosomes per cell and the number of *rrn* genes per cell, using the relationship $i_{rrn} = N_r \cdot (\ln 2/\tau)/N_{rrn}$.

[t] The nucleotide distance between ribosomes on mRNA was calculated from data in this table and the relationship $R_m/N_r = r_m \cdot t_m/(\beta_r \cdot N_r)$, where R_m is the amount of mRNA and t_m is the average functional life of mRNA, assumed to be 1.0 min (5, 25).

[u] The number of translations per mRNA was calculated from data in Table 2 and this table using the relationship $N_{trans} = 3 \cdot (dP/dt)/r_m$. The factor 3 in the numerator is the coding ratio, i.e., 3 mRNA nucleotides per amino acid residue.

[v] The *C* period was determined from age-fractionated cultures (70), synchronized cultures (13), flow cytometric data (128), and perhaps most accurately in nonsynchronous exponential cultures by measuring the increase in the amount of DNA in the culture after treatment with rifampin or chloramphenicol (26). The values obtained by these different methods agree to within 10%. The values shown are considered to be the best average of the reported data.

[w] Like the *C* period, the *D* period was determined in age-fractionated and synchronized cultures, as well as from flow cytometric data (13, 70, 129). The *D* period was also determined by measuring the increase in cell number after treating exponential cultures with sodium azide; this treatment stops replication, but does not prevent the division of cells that were already in the *D* period at the time of the replication stop (14).

The levels of ppGpp listed in Table 3 are seen to decrease from 55 to 10 pmol per OD_{460} when the growth rate increases from 0.6 to 2.5 doublings per h. At the same time, the transcription from rRNA and tRNA genes, expressed as the fraction of the total instantaneous rate of transcription, r_s/r_t, increases from 40 to 85%. The parameters ψ_s and r_s/r_t both express the same ppGpp-correlated partitioning of RNA polymerase; the difference between these two parameters reflects the difference in the chain elongation rates for rRNA and mRNA. Since rRNA chains grow faster than mRNA chains, it follows that for equal numbers of polymerase-transcribing stable RNA and mRNA genes ($\psi_s = 0.5$), the rate of stable RNA synthesis must be somewhat greater than the rate of mRNA synthesis (i.e., $r_s/r_t > 0.5$).

Rates of stable RNA and mRNA synthesis per cell. The rate of stable RNA synthesis was calculated from the amount of RNA, determined from its UV absorbance (Table 2). The rate of mRNA synthesis was found from its relative rate ($r_m/r_t = 1 - r_s/r_t$) and the absolute rate of stable RNA synthesis. It is seen that the stable RNA synthesis rate per cell, in particular, increases dramatically with growth rate, which accounts for the higher RNA content in rapidly growing bacteria (see Fig. 1). The same relationships are apparent when rates of stable RNA and mRNA synthesis are expressed per amount of protein (Fig. 2e).

Chain elongation rates of stable RNA and mRNA. Whereas the chain elongation rate of stable RNA is independent of the growth rate and equal to 85 nucleotides per s (99, 120, 124, 139), the chain elongation rate of mRNA appears to increase somewhat and approaches a maximum value of 55 nucleotides per s at high growth rates (19, 139). The reason for this velocity difference is not known, although it may be related to polymerase pausing or stuttering during chain elongation. The leader regions of *rrn* transcription units are known to contain strong Nus factor-dependent antitermination sites (85, 103). As a consequence, transcripts initiated at *rrn* promoters are able to transcribe through Rho protein-dependent transcription terminators and can reverse transposon-induced polarity (1, 127). Thus, for rRNA transcripts this pausing or stuttering may be minimized by the antitermination state of the RNA polymerase and account for the increased stable RNA chain elongation rate.

Ribosome Synthesis and Function.

Ribosomal components and their control. The ribosome consists of three species of RNA (16S, 23S, and 5S) and 52 species of protein. The three rRNAs are processed from a 35S primary transcript derived from seven unlinked *rrn* transcription units. The 52 different ribosomal proteins (r-proteins) are encoded by genes in about 20 different transcription units located at 14 different positions on the *E. coli* chromosome (4).

The primary regulation of ribosome synthesis is most likely the ppGpp-correlated partitioning of RNA polymerase which, at a given concentration and activity of RNA polymerase, sets the rate of transcription of rRNA and tRNA genes (36, 119; see also Fig. 1). However, even in the absence of ppGpp in bacterial strains deleted for both ppGpp synthetases (141), the stable RNA gene activity increases with increasing growth rate, apparently as a result of only an increase in the concentration of active RNA polymerase (74). There is the additional possibility that ppGpp might also influence either directly or indirectly the transcription of r-protein operons within the mRNA sector to achieve an approximate balance between the production of the rRNA and r-protein components of the ribosome (40, 49, 50). This is the transcriptional control of r-protein operons (35, 42, 63, 87, 89). It is clear from Fig. 1 that the promoter activities of rRNA operons and of r-protein operons do not respond coordinately to changes in the steady-state growth rate. Coordination is neither expected nor required if the ratio of r-protein mRNA to total mRNA is important in determining α_r.

A second mechanism in addition to the transcriptional control, used to accurately balance or fine tune the translation frequency of the 20 different mono- and polycistronic r-protein mRNAs to the availability of free rRNA, involves an autogenous translational control: specific regulatory r-proteins bind to the leader regions of their own mRNAs and inhibit further translation when they are not rapidly incorporated into assembling ribosomes (47, 55, 78, 88, 105).

During exponential growth at moderate to fast rates, turnover of ribosomal components appears to be negligible. During slow growth, some excess of newly made rRNA is degraded (63, 106); this results in a slight increase in the proportion of tRNA to rRNA during slow growth (124).

r-Protein synthesis. A measure for the synthesis of r-protein is its proportion of total protein, α_r. Values of α_r have been determined (i) from the protein content of ribosomes and (ii) from the RNA-to-protein ratio. Both methods give essentially identical values. The proportion of r-protein increases with growth rate from 9% at 0.6 to 21% at 2.5 doublings per h. It has been reported that the r-protein mRNA synthesis per total RNA synthesis rate is nearly invariant with growth rate, but, when expressed as a fraction of the rate of (total) mRNA synthesis, it increases with growth rate like α_r or rRNA synthesis (Fig. 1; 42, 63, 89). These observations support the idea that, to a first approximation, r-protein production is matched to rRNA production at the level of transcription.

Ribosomes and tRNA per cell. Given that r-protein matches rRNA and that rRNA and tRNA are synthesized in constant proportions, corresponding to nine tRNA molecules per 70S ribosome (37, 54, 124, 143), the numbers of ribosomes and of tRNA molecules per cell were calculated from the total amount of RNA. In the growth range considered, the number of ribosomes per average cell was seen to increase 10-fold from 6,700 to 71,000. This reflects both an increasing ribosome concentration (ribosomes per OD_{460} unit of cell mass) and increasing cell size (OD_{460} units per cell). For achieving rapid growth, only the ribosome concentration is relevant.

Ribosome activity. The fraction of ribosomes engaged in peptide chain elongation at any instant, β_r, has been estimated from the ribosome content of polysomes and was found to be about 80% and independent of the growth rate (57). Since the assembly and maturation of ribosomes takes about 5 min (86), it appears that, at least in fast-growing bacteria, the major portion of inactive ribosomes are particles in the final stages of ribosome assembly.

Peptide chain elongation rate. The peptide chain elongation rate has been estimated (i) from pulse-labeling kinetics of nascent polypeptides of given length (111, 142) and (ii) from the first appearance of β-galactosidase activity after induction (33, 61, 110, 111, 142). These measurements indicate that the peptide chain elongation rate increases with growth rate from about 13 to 20 amino acid residues per s between 0.6 and 2.5 doublings per h. These values are in agreement with the values calculated in Table 3 from the RNA-to-protein ratio under the assumption that 80% of the ribosomes are active at any instant.

What limits the peptide chain elongation rate during slow growth? During fast growth when the peptide chain elongation rate is maximal (20 amino acids per s; Table 3), ribosomes seem to be saturated with substrates (elongation factor Tu-GTP-aminoacyl-tRNA ternary complex), implying that the peptide chain elongation rate is limited by the rate of peptide bond formation or ribosome translocation on the mRNA. During slow growth, the (submaximal) peptide chain elongation rate is probably limited by the extent of tRNA charging (i.e., by the ratio of charged to uncharged tRNA) and to some extent by the types of codons being employed, rather than by the absolute concentration of charged tRNA. This is suggested by the observation that in a nutritional shift-up from a minimal medium to an amino acid-supplemented medium the protein synthesis rate per average ribosome increases immediately in a stepwise fashion, before the concentrations of tRNA and EF-Tu have increased (20). During further postshift growth, the concentration of tRNA, and thus presumably also of charged tRNA, increases severalfold (e.g., about threefold in a shift from succinate minimal to glucose amino acids medium) without further increasing ribosome activity (126). It is also unlikely that ppGpp binds to and thereby inhibits elongation factor Ts function during slow growth since the peptide chain elongation rate was unaffected in a ribosome control mutant with 10-fold reduced level of ppGpp (90).

It is conceivable that in poor media, the greater proportion or abundance of mRNAs with hard-to-read codons might contribute to the reduced peptide chain elongation rate. It is well established that the utilization of codons in the genes of *E. coli* is not random (113, 123, 138). Genes expressed from strong promoters tend to contain a higher proportion of major codons, whereas genes expressed from weak promoters contain a lower proportion of major codons. With some exceptions, major codons are recognized by abundant tRNA species and

minor codons are recognized by rare tRNA species (76). However the concentration of minor tRNAs is probably not limiting the rate of peptide chain elongation. Instead, each codon probably has a specific transit time for progressing through the A- and P-sites of the ribosome that depends upon the physical-chemical nature of the tRNA-mRNA (anticodon-codon) interaction. As an example, the GAA and GAG glutamic acid codons are both recognized by the abundant $tRNA^{Glu}_2$, but decoding of the prevalent GAA triplet involving strict Watson-Crick base pairing occurs threefold more rapidly than the decoding of the GAG triplet involving wobble base pairing (110, 131, 132).

Ribosomal gene dosage and gene activity. There are seven *rrn* genes on the *E. coli* chromosome; most of them map near the chromosomal origin of replication (4). The actual number of *rrn* genes per (average) cell is much greater than 7, ranging from 12 to 36, depending on the extent of chromosome branching (see Fig. 1). From this number and the rate of rRNA synthesis, the rate of initiation of rRNA chains at each *rrn* gene was calculated and was found to increase with increasing growth rate from 4/min to 61/min. These are the average values in an exponential culture. Measurements of DNA and RNA synthesis rates in age-fractionated cultures indicate that replication causes the number of *rrn* genes to fluctuate nearly twofold during the cell cycle without abrupt or concomitant changes in the rate of rRNA synthesis (36). Instead, the rate of rRNA synthesis increases by a factor of 2 in a slow and continuous manner, without perturbation, as cells progress through the division cycle; this pattern is followed regardless of when the *rrn* genes are replicated (36, 37). This implies that the rate of transcription initiation at *rrn* genes changes (i.e., decreases) nearly twofold at the time the *rrn* genes are duplicated. Accordingly, the average rate of 61 initiations per min per gene means a fluctuation from about 40 to 80 initiations per min per gene during the cell cycle for a bacterium with a 24-min doubling time (actually the fluctuation is somewhat less because rRNA genes are not all clustered at exactly the same map position). Therefore the copy number of *rrn* genes does not limit the rate of rRNA synthesis under conditions of exponential growth. In addition, if there are up to 80 initiations per min per gene in rapidly growing bacteria, the time for the formation of the open promoter complex must be less than 1 s.

TABLE 4 Stoichiometric content of transcription-translation proteins in *E. coli*

Protein	Mol wt (10^3)	α_i[a] (t = 40 min) (%)	Molecules (τ = 40 min)		Reference(s)
			Per OD_{460} (10^{12})	Per ribosome	
r-Protein	850	13.5	10.2	1.00	38, 44
L7/L12	12	0.81	40.8	4.00	134
EF-Tu	42	5.55	55.1	5.40	112
EF-G	84	1.66	8.2	0.80	112
EF-Ts	31	0.13	1.8	0.18	112
IF1	8	0.04	2.5	0.25	75
IF2	115	0.52	3.1	0.30	75
IF3	20	0.07	2.0	0.20	75
Leu S	100	0.12	0.5	0.05	112
Phe S-β	94	0.21	1.0	0.10	112
Lys S	58	0.11	0.8	0.08	112
Arg S	58	0.08	0.6	0.06	112
Gly S	77	0.17	0.9	0.09	112
Val S	106	0.14	0.6	0.06	112
Glu S-β	48	0.10	0.9	0.09	112
Ile S	107	0.24	1.0	0.10	112
Phe S-α	36	0.11	1.2	0.12	112
Gln S	61	0.11	0.8	0.08	112
Thr S	65	0.09	0.6	0.06	112
RNA polymerase β	150	0.52	1.4	0.14	112
RNA polymerase α	39	0.37	3.8	0.37	112
RNA polymerase, core	375	1.30	1.9	0.19	125

[a] α_i, synthesis rate of the protein as a percentage of total protein synthesis rate.

These conclusions have been further corroborated by the observation that the rRNA synthesis rate is not reduced in bacteria with a mutational defect in the control of chromosome replication that leads to a 40% reduction in the concentration of all genes, including *rrn* genes (28). Furthermore, up to three *rrn* genes may be deleted from the *E. coli* chromosome without much change in the growth rate, again suggesting that the *rrn* gene dosage does not limit the rate of rRNA synthesis (31; it is to be noted, however, that contrary to the authors' interpretation, we believe that the rRNA synthesis rate per gene was not suitably measured because of the use of inappropriate reference units).

Translation frequency of mRNA. With increasing growth rate the average mRNA becomes more and more crowded with ribosomes, i.e., the average spacing of ribosomes on mRNA decreases from 120 nucleotides to 60 nucleotides in the range of growth rates considered. Here again, there is no indication that mRNA is a limiting factor for protein synthesis. Immediately after a nutritional shift-up the concentration of mRNA decreases temporarily because the increased rate of rRNA synthesis occurs partly at the expense of mRNA synthesis; at the same time, the protein synthesis rate increases (45, 102, 126). The increased spacing of ribosomes during slow growth could potentially cause some mRNA instability or premature termination of transcription (polarity), but whether this is indeed the case has not been established.

Component proteins of the transcription-translation apparatus. The protein composition of the ribosome is essentially invariant with the growth rate, and each of the 52 different r-proteins is present in one copy per 70S particle (38, 68). The only exception to this is protein L7/L12, which is present in four copies per ribosome (134). This implies that the synthesis rate of each r-protein is strictly coordinate with the synthesis of rRNA and also tRNA at growth rates above 0.5 doublings per h. At slower growth rates there appears to be a slight excess in the synthesis rate of stable RNA (62, 63, 106); the excess rRNA is rapidly degraded, whereas the tRNA accumulates.

The synthesis rates of other components of the transcription-translation apparatus also appear to be subject to growth rate-dependent regulation (59, 65, 75, 112, 125). These components include translation initiation and elongation factors, the subunits of RNA polymerase, and the aminoacyl-tRNA synthetases (Table 4). From the available data it seems clear that the concentration of all these components increases with growth rate, but the increases might not be—and for at least some of the proteins such as RNA polymerase, for example, are not—strictly parallel with the increase in ribosome (and r-protein) concentration. In Table 4 we list the proteins which have been examined in this context and give the α_i values for each (i.e., the synthesis rate of the protein as a percent of the total protein synthesis rate) at a growth rate of 1.5 doublings per h (τ equals 40 min). In addition, the numbers of molecules of each protein per unit of mass and per ribosome are also indicated. In compiling the information in this table we have had to reinterpret or extrapolate some of the original measurements in the cited references. If the synthesis of these proteins were strictly coordinate with synthesis of ribosomes, the listed number of molecules per ribosome would remain constant and not change with changes in the steady-state growth rate.

The r-protein operons also encode the genes specifying the α, β, β', and σ subunits of RNA polymerase and the protein synthesis elongation factors Tu, Ts, and G (for review see reference 88). There is a second gene for Tu on the *E. coli* chromosome which is not in an r-protein operon; presumably two copies of this gene are required to produce the six molecules of Tu per ribosome at high growth rates. The β and β' RNA polymerase subunit genes, although cotranscribed with the L10 and L12 r-protein genes, are regulated somewhat independently by a transcription attenuator located between the upstream r-protein genes and the downstream RNA polymerase genes (see RNA polymerase synthesis and function, above).

There are about nine tRNA molecules per ribosome in exponentially growing *E. coli*, and this ratio shows little variation for growth rates above 0.5 doublings per h (Table 3). Since the peptide chain elongation rate approaches 20 amino acids per s, each tRNA is required to cycle through the ribosome on average about two times per second. Ikemura (76) has quantitated over 70% of the total tRNA population into 26 separate species, at least one for each amino acid except for proline and cysteine. For each of these 18 different amino acids there is at least one major isoacceptor which is present at a molar ratio of 0.15 to 0.60 copy per ribosome. The aminoacyl-tRNA synthetases are present at about 0.1 copy per ribosome; each

synthetase molecule is therefore required to aminoacylate about 10 molecules of its cognate tRNA every second to sustain protein synthesis.

The elongation factor Tu is required for the GTPase-dependent deposition of aminoacylated tRNA into the A-site of the translating ribosome. The charging level of tRNA is about 75 to 90%. There are between two and three tRNA molecules bound to each translating ribosome (98, 116). The six copies of Tu are available for ternary complex formation with GTP and the remaining aminoacylated tRNAs. The concentration of the ternary complex required to initiate the process of amino acid addition on the translating ribosome is thus maximized.

During periods of amino acid insufficiency the synthesis of r-protein, like that of rRNA and tRNA, is subject to stringent regulation; this control is exerted at the level of transcription (40, 49, 50). Many of the genes that are cotranscribed with r-protein genes are also, as expected, stringently regulated. These include the genes encoding the elongation factors G, Tu, and Ts (8, 60, 115). In contrast, the genes encoding the β, β', and σ subunits of RNA polymerase are not stringently regulated (8). The nonstringent regulation of transcription of the β and β' genes is mediated by control at the attenuator in the L10 (*rplJL rpoBC*) operon (96). In the case of the *rpoD* (σ) gene, a new promoter signal is utilized (C. Gross, personal communication). With respect to aminoacyl-tRNA synthetases, the data on their stringent regulation are equivocal (8).

DNA Replication and Cell Division.

Chromosome replication time. The *C* period is the time interval required for the replication forks to move from the origin (*oriC*, at 84 min on the *E. coli* genetic map) to the terminus (*terC*, approximately at 36 min on the genetic map; 91). Pulse-labeling of the terminus in cells with synchronized replication has indicated that both replication forks created at every initiation event move with equal speed (65 kilobases/min for wild-type strains) clockwise and counterclockwise, respectively, with very little variation from cell to cell (9, 10). In addition, the time intervals between consecutive replications of any given section of the chromosome are very constant and equal to the mass doubling time (83, 97, 101). This suggests that both the times between consecutive initiations of rounds of replication and the replication velocities themselves are constant within a cell population.

The *C* period has been measured by Helmstetter and Cooper (70) in age-fractionated cells. Due to the considerable variability from cell to cell in the duration of the *D* period (see below), the initiation age, termination age, and interdivision interval all vary (12). This makes the determination of *C* (and *D*) from age-fractionated or synchronous cultures somewhat inaccurate. The *C* period has also been measured in exponential cultures (i) from the relative frequencies of genes at given map locations (equation 9, Table 5 below; reference 22) and (ii) from the increase in DNA after stopping of initiation (26, 114, 144). Cooper and Helmstetter (32) estimated that the *C* period was constant (41 min) for growth rates above 1 doubling per h and increased in proportion to τ at lower growth rates. Measurements of the increase in DNA after a replication stop (26) suggest that the *C* period decreases gradually with increasing growth rate, approaching a value of 40 min in rapidly growing bacteria (Table 3).

Chromosome segregation and cell division. The *D* period is the time between termination of a round of replication and the following cell division. The cell division is believed to require the action of a protein synthesized at the time of termination of replication (79). During the *D* interval the completed chromosomes are segregated. The length of time between the completion of replication and the onset of constriction is subject to a stochastic process (13, 14). This results in a (non-Gaussian) fluctuation in the length of the *D* interval, which is the major factor contributing to cell cycle variability including (i) the variability of the initiation age (the time after cell division at which a round of chromosome replication is initiated) and (ii) the variability of the time intervals between consecutive divisions (12).

Helmstetter and Cooper (70) estimated the average *D* to be about 22 min for growth rates above 1 doubling per h and to increase as a constant fraction of the doubling time for growth rates below 1 doubling

per h. The average D period has also been determined in exponential cultures from the increase in the cell number after a replication stop by thymine starvation or by the addition of sodium azide (14). Cells that do not terminate replication due to an experimentally induced replication stop will not divide, whereas cells that have already terminated and have made termination protein, i.e., cells in the D period, divide once (79). These experiments suggest that the D period decreases from about 30 min during slow growth to about 22 min during rapid growth.

The average C and D intervals have also been obtained by the method of flow cytometry, which measures the distribution of the amounts of DNA (labeled with a fluorescent dye) per cell in exponential cell populations (frequency of cells as a function of DNA per individual cell). In this manner, values of C equal to 42 min and D equal to 22 to 24 min were found for *E. coli* B/r A during balanced growth with a 27-min doubling time (129).

At a given doubling time, the C and D periods determine the initiation age, a_i, and the termination age, a_t (Fig. 1; see Table 5 below). Depending on the values of the three parameters, C, D, and τ, rounds of replication may be initiated at the beginning, in the middle, or near the end of the cell cycle (Fig. 1). If initiation occurs on average at the beginning of the cell cycle, it means that initiation actually occurs shortly before division in some cells of the population and shortly after in others.

In thymine-requiring bacteria, where the DNA replication velocity can be altered by changing the thymine concentration in the growth medium, the C period, and thus the initiation age, can be experimentally changed. This has no effect on cell growth rate or on the control of replication initiation. When the C period is extended, the time of the cell division, which occurs C plus D min after initiation, is delayed. As a consequence, cells are larger than normal, but their ribosome concentration is unaltered (28).

Replication initiation control depends on P_O, i.e., the amount of protein per origin. Changes in P_O (e.g., by mutation) do not affect the initiation age (24). This apparent paradox reflects the fact that a change in the initiation time (without a change in C and D) causes an equal change in the time of division so that the initiation age remains unaltered.

Macromolecular Composition during Growth at Different Temperatures

At 20, 25, 30, 35, and 40°C the growth rates of *E. coli* B/r in glucose minimal medium were 0.41, 0.65, 0.91, 1.18, and 1.35 doublings per h, respectively. At these temperatures the rRNA chain elongation rates were 30, 45, 59, 76, and 103 nucleotide residues per s, and the peptide chain elongation rates were 5, 8, 11, 14, and 16 amino acid residues per s, respectively (120). Most of the other physiological parameters are essentially unaltered by a change in temperature. In particular, the relative proportions of mRNA and stable RNA synthesis are the same, suggesting that the chain elongation rates of stable RNA and mRNA have equal temperature coefficients. The growth rate is expected to change as the square root of the changes in the product of the peptide and RNA chain elongation rates (equation 19, Table 6 below), which is, indeed, observed.

The C period changes with temperature in proportion to the doubling time (i.e., the ratio C/τ is constant; 24, 58), which implies identical replication fork patterns at different temperatures. The chain elongation rates for DNA, RNA, and polypeptides have thus about equal temperature coefficients, which in the absence of further regulation results in a temperature independence of the macromolecular cell composition. During the first 30 min after a temperature upshift, extensive temporary changes in the macromolecular synthesis rates have been observed (e.g., reference 120) and a new RNA polymerase sigma subunit is induced (67). These temporary perturbations constitute the heat shock response and reflect active regulation and adjustment to the postshift temperature.

MATHEMATICAL DESCRIPTION OF CELL COMPOSITION AND GROWTH

Cell Composition as a Function of the Culture Doubling Time

A number of equations have been reported that describe the macromolecular composition of an average cell in an exponential culture as a function of the culture doubling time and five additional parameters: the C and D periods, protein per origin (P_O), ribosome activity (β_r), and peptide chain elongation rate (c_p) (see History, above; 27, 32, 51, 122). These equations, reproduced in Table 5, are useful for work dealing with the cell composition and have been used for the calculation of many of the parameters in Table 3. Additional equations in Table 5 can be used to calculate the copy number of genes per cell or per genome as a function of their map location. All equations in Table 5 follow from the definitions of their constituent parameters without special, simplifying assumptions or hypotheses.

Age Distribution and the Concept of the Average Cell

The *average number of a component per cell* has to be distinguished from the number of that component *per average cell.* The first is obtained as the number per unit volume of culture, divided by the number of cells in that volume. This quotient can be any noninteger number. The latter refers to a particular *average cell,* which is defined by the fact that 50% of all cells in the population are younger and 50% are older. Because young cells are more frequent than old cells in an exponential population, the average cell has an age of 0.41, rather than 0.50. The number of any component in the average cell is always an integer; for example the number of chromosome replication origins in the average cell is equal to 2, 4, or 8, depending on the growth rate (see Fig. 1). In contrast, the average number of origins per cell (given by equation 7 in Table 5) may have any noninteger value, such as 2.43 for a growth rate of 1 doubling per h (Table 2), implying that some cells in the population have four origins, while others have only two. These average numbers of components per cell are identical to the values calculated from the formulas in Table 5.

To calculate a population average, the equation for the "ideal age distribution" (135) (meaning that all cells divide in exactly equal time intervals) has been used in the past, with integration over different age intervals. For example, the Cooper-Helmstetter equation and Donachie's equation for the average amounts of DNA and protein, respectively, per cell (equations 3 and 1 of Table 5) were originally derived under the assumption of an ideal age distribution. However, a reexamination of these equations has indicated that they are independent of any assumptions, including the assumptions of an ideal age distribution and of synchronous initiation at all origins in the cell at a given initiation age (15, 18). The formulas in Table 5 give correct values irrespective of the age distribution. In fact, the cell cycle variability has no effect on the average cell composition.

The conclusion, that the cell cycle variability has no effect on the composition and growth parameters, has been disputed by Alberghina and Mariani (2). These authors have not distinguished the doubling time, τ, defined as cell number doubling time in an exponential culture (equal to the mass doubling time), from the average interdivision interval, denoted by τ. The latter may be determined from growth curves of synchronous cultures (13) and depends on the particular subpopulation of cells for which individual division intervals are measured. Although synchronous cells have the same division age, they are generally somewhat out of phase with respect to their replication cycles. This means that a zero-age population may contain a large number of subpopulations in which the cells are in step, only in different phases, with respect to their last round of chromosome replication. Each of these subpopulations (with different phase relationships between their replication age and division age) would give a different synchronous growth curve and a different average interdivision interval despite an equal mass doubling time (12). For these reasons, τ and τ (and, similarly, C and C, and D and D) must be distinguished in theory (15, 27); only with C, D, and τ are the relationships in Table 5

strictly valid. However, the extent of variability in the cell cycle is such that the differences between τ and τ, etc., amount to only a few percent (12) and, in practice, are negligible.

TABLE 5 Equations relating the cell composition in exponential cultures to basic cell cycle parameter[a]

Parameter	Symbol	Equation	Equation no.	Reference(s)
Protein/cell	P_C	$P_C = P_O \cdot 2^{(C+D)/\tau}$	1	51
RNA/cell	R_C	$R_C = K'(P_{O\alpha cp})(1/\tau)2^{(C+D)/\tau}$, where $K' = (\text{nucl./rib}) \cdot \ln 2/[f_s \cdot (1 - f_i) \cdot \beta_r \cdot 60]$	2	27
DNA/cell	G_C	$G_C = [\tau/(C \cdot \ln 2)] \cdot [2^{(C+D)/\tau} - 2^{D/\tau}]$	3	32
Mass/cell	M_C	$M_C = k_1 \cdot P_C + k_2 \cdot R_C + k_3 \cdot G_C$, where: $k_1 = 1.35 \cdot 10^{-18}$ OD_{460} units per amino acid residue $k_2 = 4.06 \cdot 10^{-18}$ OD_{460} units per RNA nucleotide residue $k_3 = 3.01 \cdot 10^{-11}$ OD_{460} units per genome equivalent of DNA	4	27
Peptide chain elongation	c_p	$c_p = K'/[(R/P) \cdot \tau]$	5	44, 122
Ribosomal protein/total protein	α_r	$\alpha_r = (R/P) \cdot [(\text{aa/ribosome}) \cdot f_s \cdot (1-f_i)/(\text{nucl./rib})]$	6	44, 122
Origins/cell	O_C	$O_C = 2^{(C+D)/\tau}$	7	15, 23
Termini/cell	T_C	$T_C = 2^{D/\tau}$	8	15, 23
No. of gene *X*/cell	X_C	$X_C = 2^{[C(1-m')+D]/\tau}$, where: m' = map location of gene *X* relative to location or replication origin $= (m + 16)/50$ for map locations (m) between 0 and 36 min $= (84 - m)/50$ for map locations between 36 and 84 min $= (m - 84)/50$ for map locations between 84 and 100 min	9	15, 23
Replication forks/cell	F_C	$F_C = 2 \cdot [2^{(C+D)/\tau} - 2^{D/\tau}$	10	15, 23
Origins/genome	O_G	$O_G = (C/\tau) \cdot \ln 2/(1-2^{-C/\tau})$	11	15, 23
No. of gene *X*/genome	X_G	$X_G = (O/G) \cdot 2^{-m'O\tau}$	12	15, 23
Initiation age	a_i	$a_i = 1 + n - (C + D)/\tau$ where n is the next lower integer value of $[(C + D)/\tau]$; i.e., $n = \text{int}[(C + D)/\tau]$	13	32
Termination age	a_t	$a_t = 1 - D/\tau$	14	32
Origins per cell at initiation	O_i	$O_i = 2^n$; for a definition of n, see equation 13	15	32
Cell mass after division (a_0)	M_d	$M_d = M_C/(2 \cdot \ln 2)$	16	18
Cell mass at initiation (a_i)	M_i	$M_i = M_d \cdot 2^{a_i}$	17	18

[a]See Tables 1 and 2 for definitions.

Cell Composition at a Defined Cell Age

In some instances the cell composition at certain cell ages becomes important; in particular, at the time of cell division (either shortly before or shortly after) or at the time of initiation of chromosome replication. In these cases, it is also not necessary to use the age distribution formula. Instead, the following relationships can be used. (i) The average amount of a component in the subpopulation of zero-age cells (immediately after division) is $1/(2 \cdot \ln 2)$ times the average amount of that component in the population as a whole (obtained as described above). Correspondingly, the factor to obtain the average amount of the component in the cells immediately before division has twice that value, i.e., $1/(\ln 2)$. (ii) The amount of protein (or cell mass) per replication origin in a cell at the time of initiation ("initiation mass" defined by Donachie [51]) is $P_O/\ln 2$ (or $M_O/\ln 2$), where P_O (or M_O) is the total protein (or cell mass) divided by the total number of chromosomal replication origins in a unit volume of exponential culture. (Table 2 shows only P_O, but M_O may be calculated from the data in the Table.) For the mathematical relationships dealing with the age distribution, see references 12, 15, and 16.

Parameters Determining Bacterial Growth Rate

In the preceding discussion, the cell composition was expressed as a function of the growth rate. This suggests that the composition is determined by the growth rate. In reality, the nutrients and other components in the medium, together with genetically determined structural and kinetic constants of cellular

components, determine both the biochemical reaction rates (and thus the growth rate) and the cell composition. It seems likely that only a few physiological parameters limit cell growth, whereas other physiological parameters and most of the reaction rates and concentrations in a bacterium are probably not growth limiting.

The DNA concentration is not growth limiting. Maaløe and Kjeldgaard (94, 95) have discussed the idea that the amount of protein per DNA is constant and that the amount of RNA per DNA increases in direct proportion to the growth rate. These relationships seem to suggest a limitation by DNA, for example, that mRNA synthesis is limited by DNA and protein synthesis is limited by mRNA, which then results in a constant ratio of protein to DNA. Maaløe (94) argued that the apparent and exact proportionality of the ratio of RNA to DNA reflected the control of growth. Similarly, Koch (82) and Daneo-Moore and Schockman (34) have used rate constants of RNA synthesis per DNA in models for the control of RNA synthesis or growth. However, a DNA replication (initiation) mutant which has a reduced DNA concentration and therefore an increased ratio of RNA to DNA at all growth rates (because the denominator, DNA, is reduced) has an unaltered growth rate (24, 28, 29). The reduced DNA concentration is the result of an increased initiation mass (protein per *oriC*, P_O). This mutant shows that RNA synthesis and growth are not normally limited by the concentration of DNA; in the mutant, the ratio of RNA to DNA is no longer exactly proportional to the growth rate. Moreover, the amount of protein per DNA is generally not constant, even in wild-type cells (see Fig. 2b).

TABLE 6 Basic parameters determining the bacterial growth rate[a]

Parameter	Symbol	Equation[b]	Equation no.
Growth rate (doublings/h)	μ	$\mu = (60/\ln 2) \cdot (N_r/P) \cdot e_r$, where the ribosome efficiency, $e_r = \beta_r \cdot c_p$	18
Growth rate (doublings/h)	μ	$\mu = (60/K) \cdot [\psi_s \cdot \alpha_p \cdot \beta_p \cdot \beta_r \cdot c_s \cdot c_p]^{0.5}$, where $K = \ln 2 \cdot [(\text{nucl./prib})(\text{aa/pol})/(1-f_i)]^{0.5}$	19

[a] c_s and c_p in these equations should be expressed as rates per minute to obtain the growth rate in doublings per hour. For definitions, see Table 1. (The equation 19 is from reference 11.)

[b] For a definition or explanation of symbols, see Tables 1 and 3.

The ribosome concentration (measured as number of ribosomes per protein) and the protein synthesis rate per ribosome are growth limiting in any living cell whose protein turnover is negligible. These two factors determine the exponential growth rate (equation 18, Table 6). Equation 18 is identical to the one used by Schleif (122) to evaluate the RNA-to-protein ratio as a function of growth rate (see also equation 5, Table 5). In his case, however, the growth rate, μ, was the independent variable and *R/P* was the dependent variable. By making μ the dependent parameter, we have here exchanged the roles of the two variables.

For any growth equation to be meaningful, the parameters must be constant in time. For example, in equation 18 (Table 6), both the ribosome concentration and activity must be constant. If they changed, μ would have a changing value and growth would not be exponential. A constant and given ribosome concentration results from the regulation of ribosome synthesis, which in turn involves the regulation of RNA polymerase synthesis and its partitioning into rRNA and mRNA-synthesizing enzyme (see above). These additional concepts have been taken into account in the more complex growth equation 19 (Table 6), which contains six factors: RNA polymerase concentration (α_p), RNA polymerase activity (β_p), partitioning of active RNA polymerase into stable RNA and mRNA-synthesizing enzyme (ψ_s), ribosome activity (β_r), and the chain elongation rates for stable RNA and polypeptides (c_s, c_p).

In equation 19 the parameters must again be constant in time to produce exponential growth. In the preceding discussion (see above, Observed Cell Composition of *E. coli* B/r), the constancy of physiological parameters during exponential growth was implicit in the definition of exponential growth, but if one asks for the conditions that lead to exponential growth, then this constancy cannot be taken for granted. A full explanation of why these parameters have certain, constant values under given growth conditions would solve the problem of growth control. Growth equations such as those in Table 6 identify growth-limiting parameters and predict their effect on the growth rate

Optimal Cell Composition for Maximal Growth

Maaløe and Kjeldgaard (94, 95) have pointed out that the protein synthesis rate per average ribosome in bacteria is constant and presumably maximal under most growth conditions and, further, that this constancy is economically advantageous for the cell. Since ribosomes are more expensive than their substrates, they should always work at their maximum rate and therefore be saturated with substrates. Thus, the increased demand for protein synthesis at higher growth rates can only be achieved by increasing the ribosome concentration, since the rate of protein synthesis per ribosome is already maximal. Similarly, the increases in α_r and ψ_s with increasing growth rate can then be understood as consequences of the constant ribosome function. These arguments try to explain the changing cell composition as an expression of an optimization principle which allows the cell to achieve maximum growth at a minimum expenditure of energy.

Ehrenberg and Kurland (53) have theoretically analyzed how metabolic pathways should be designed for maximum energy efficiency. With regard to ribosomes, they concluded that an optimal utilization of energy would be achieved if both the substrate and ribosome concentration were to increase with increasing demand for protein synthesis (substrates for the ribosome are the different elongation factor Tu-aminoacyl-tRNA-GTP ternary complexes.) This can be understood as follows. At high ribosome concentrations, the substrate pools, even at saturating concentrations, would represent only a small fraction of the total mass of the protein-synthesizing system. At low ribosome concentrations the same pool would constitute a greater fraction of the total system mass, and the energy required to produce that substrate pool would no longer be negligible. The cells would then save energy by reducing the concentration of substrates, especially when this concentration is above the K_m for substrate binding, such that substantial reductions in substrate concentrations can be compensated for by only small increases in ribosome number. In support of the conclusion of Ehrenberg and Kurland, it should be noted that the ribosome function (c_p) does, indeed, increase with growth rate (Table 3). Also, the concentrations of tRNA and elongation factors Tu and Ts are not constant but increase in proportion to the ribosome concentration (Table 4). If these concentrations were above the K_m at fast growth rates, their reduction during slow growth should save energy.

It is likely that energy efficiency has played a major role in the evolution of the regulatory parameters that determine the macromolecular composition and growth rate of the cell. In addition, other principles, like rapid adaptability to a changing growth environment, have evolved at the expense of energy efficiency. In some instances an inefficiency might be a necessary byproduct of control mechanisms; for example, the "stuttering" and pausing RNA polymerase might be the result of controls that depend on the secondary structure of mRNA, like RNA chain termination, attenuator control, or control of mRNA stability.

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